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Do spermathecal morphology and inter-mating interval influence paternity in the polyandrous beetle *Tribolium castaneum*?

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Summary

In polyandrous insects, postcopulatory sexual selection is a pervasive evolutionary force favouring male and female traits that allow control of offspring paternity. Males may influence paternity through adaptations for sperm competition, and females through adaptations facilitating cryptic female choice. Yet, the mechanisms are often complex, involving behaviour, physiology or morphology, and they are difficult to identify. In red flour beetles (*Tribolium castaneum*), paternity varies widely, and evidence suggests that both male and female traits influence the outcome of sperm competition. To test the role of spermathecal morphology and of sperm storage processes on the outcome of sperm competition, we mated each of 26 virgin females with two males, one of which carrying a phenotypic marker to assign offspring paternity. We manipulated the interval between mating with the first and the second male, to create different conditions of sperm storage (overlapping and non-overlapping) in the female reproductive tract. To investigate the role of sperm storage more closely, we examined the relationship between paternity and spermathecal morphology in a subset of 14 experimental females. In addition, we also characterized variation in spermathecal morphology in three different strains, wildtype, Chicago black and Reindeer. No significant influence of the intermating interval was found on the paternity of the focal male, although the direction of the difference was in the expected direction of higher last male paternity for longer intervals. Moreover, paternity was not significantly associated with spermathecal morphology, although spermathecal volume, complexity, and tubule width varied significantly and substantially among individuals in all investigated strains.

Keywords: reproduction, behaviour, sexual selection, cryptic female choice, sperm storage, confocal microscopy, *Tribolium castaneum.*

Introduction

In polyandrous species, male reproductive success depends on access to females (mating success) and on the ability to secure fertilizations against sperm from competing males (post-mating success). Post-mating success depends on male adaptations for sperm competition (Birkhead & Møller, 1998) and on female ability to favour some males over others at fertilization (cryptic female choice, Eberhard, 1996; Thornhill, 1983). Accordingly, several studies found that both male and female genotype can influence reproductive success of males in different species (Lewis & Austad, 1990; Wilson et al., 1997; Clark et al., 1999; Andres & Arnqvist, 2001; Brown & Eady, 2001; Nilsson et al., 2003). Influence of both male and female on the outcome of sperm competition suggests that the underlying mechanisms may often be complex, and involve morphological, physiological or behavioural traits of both sexes (Eberhard, 1996; Simmons, 2001). A particularly important candidate mechanism in females is control of sperm storage, i.e. the process by which inseminated sperm is uptaken or eliminated (e.g., Otronen, 1997; Neubaum & Wolfner, 1999).

It has been suggested that the size (Walker, 1980) and the complexity (Hellriegel & Ward, 1998) of sperm storage organs can increase female control of paternity. In agreement with this idea, female control of sperm storage or storage of sperm of different males in different compartments of complex sperm storage organs has been reported (chrysomelid beetles: Rodriguez, 1994, flies: Hellriegel & Bernasconi, 1999, onychophorans: Curach & Sun-nucks, 1999; spiders: Snow & Andrade, 2005).

In this study, we investigate whether complex spermathecal morphology and differential sperm storage influence the outcome of sperm competition in the red flour beetle, *Tribolium castaneum.* Males and females mate multiply in this species (Sokoloff, 1974). Male reproductive success after competitive matings varies considerably (Lewis & Austad, 1990; Bloch Qazi et al., 1998; Haubruge et al., 1999; Edvardsson & Arnqvist,

2000; Bernasconi & Keller, 2001; Pai & Yan, 2002; Bloch Qazi, 2003; Nilsson et al., 2003; Fedina & Lewis, 2004). Female influence has been reported in several studies. Paternity was found to correlate with female perception of male copulatory courtship (Edvardsson & Arnqvist, 2000) and with male olfactory attractiveness to females (Lewis & Austad, 1994). Further, experimental interference with female muscular control after mating with a single male affected the amount of sperm reaching the spermatheca (Bloch Qazi et al., 1998), suggesting that females possess mechanisms to control the amount of sperm stored.

To test whether sperm storage is also involved for cryptic female choice when females mate with more than one male, we manipulated remating intervals to interfere with sperm storage after mating with a focal male. Between 10 and 60 minutes after mating, sperm number increases in the spermathecae, while sperm are eliminated from the bursa, and thereafter sperm numbers in the bursa and spermatheca stabilize (Bloch Qazi et al., 1996). Thus, we assume that if females re-mate before storage of her first mate's sperm is completed, sperm of both males will partially mix in the bursa and the two sperm storage processes will overlap, lowering female control of sperm uptake rates. By contrast, we expected that with a longer inter-mating interval, storage of male 1 and male 2 sperm will be separate processes allowing for increased female control. Alternatively, immediate remating may allow better comparison among male phenotypes and thus result in more pronounced preference. In either case, we expected that manipulation of inter-mating interval should reveal female influence. Further, we analyzed the correlations between paternity success of focal males and spermathecal traits, to address the role of variation in spermathecal morphology as a potential predictor of male fertilization success. We also characterized intraspecific variation in spermathecal morphology for three strains.

Material and methods

Study species

The red flour beetle, *Tribolium castaneum,* is a cosmopolitan pest of stored products such as corn, wheat and rice (Sokoloff, 1974; Beeman, 2003). We used wildtype beetles (Georgia-1) and two spontaneous mutants: Chicago black (a partially dominant, autosomal cuticular colour mutant, Sokoloff et al., 1960) and Reindeer honey dipper, a dominant mutant causing antennae with enlarged terminal cubs (Dawson, 1984). Strains were obtained from the Tribolium Stock Centre (U.S. Grain Marketing Research Laboratory, Manhattan, KS). Laboratory strains kept separately for many generations may diverge (Nilsson et al., 2002). For instance, males from Chicago black strain achieve on average lower second-male paternity (P2-value, Boorman & Parker, 1976) than wildtype males when mated to wildtype females (e.g., Bernasconi & Keller, 2001). Spermathecal size may vary with overall body size. Our stocks varied in body size; elytral length (mean ± SD; precision: 0.001 mm) was 2.53 ± 0.07 mm in wildtype (N = 21), 2.39 ± 0.09 mm in Reindeer (N = 21) and 2.32 ± 0.10 mm in Chicago black (N = 25). Stock cultures were kept in 1-litre jars in a dark, ventilated rearing chamber at 30°C, 60-70% RH and standard medium (pre-treated flour; 65°C/12 h, 95% by weight and dried yeast: -20°C/12 h, 5% by weight).

The spermatheca is a muscular chamber; it normally consists of three (±1 mm) long, convoluted tubules connected to the bursa copulatrix by a common duct (Sinha, 1953; Surtees, 1960, 1961; Bloch Qazi et al., 1998; Fedina & Lewis, 2004). During mating, the male deposits a spermatophore in the bursa copulatrix and sperm are translocated to the spermatheca where they can be stored and used for fertilization for up to three months (Good, 1933; Sinha, 1953; Schlager, 1960; Surtees, 1961; Bloch Qazi et al., 1996, 1998). Males can transfer >100,000 sperm per copulation, but only about 4% reach the spermatheca, which can store roughly 7500 sperm. Thus, the spermatheca is filled to capacity after two matings and when females remate, stored sperm are partly displaced (Lewis & Jutkiewicz, 1998).

Intraspecific variation in spermathecal traits

We preliminarily examined spermathecal morphology within and between strains, using histology and confocal laser scanning microscopy (CLSM). For histology, mated females (N = 10 in each of wildtype, Reindeer and Chicago black strains) were obtained from stock cultures, anaesthetized on ice and the abdomen cut distally of the third leg pair under a dissecting microscope. The abdomina were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer for 3 h at 4°C. The osmolarity of the fixative was adjusted with sucrose to 350 mOsm. After washes in buffer the tissue was post-fixed for 2 h in 1.3% Os04 in 0.1 M S-collidine buffer. The tissue was dehydrated in 2,2-dimethoxypropane and embedded in Epon 812. Sections were cut at 1.5 μm with glass knives and stained with 3%-methylene blue (aq - w/v).

For CLSM, females (N = 5 in each of wildtype, Reindeer and Chicago black strains) from stock cultures were anaesthetized on ice and dissected in 3%-paraformaldehyde under magnification. The reproductive tract was

washed once in PBS buffer and embedded in a droplet of MOWlOL-solution (containing MOWIOL (Calbiochem), glycerol (analytical grade), Tris-buffer, l,4-diazabicyclo(2.2.2).octan (DABCO, Fluka)). As a spacer of defined depth for the cover lid we used PARAFILM "M" (American National Can). Samples were examined using a Leica TCS-SP2 confocal laser scanning microscope equipped with an 20× oil immersion objective (HC PL APO 0.70 IMM/CORR 20x), an UV-laser (351 nm) and Leica Confocal Software (Leica Microsystems Heidelberg GmbH). Images were taken in 512 × 512 pixels-format, with a zoom factor of 3.5 on average (zoom factor varied between 3.0 and 4.0 and was entered as a covariate in the analysis). The interval among optical sections in the z-stack was 0.8 μm. These settings resulted in image dimensions of 214.23 × 214.23 μm in x- and y; image dimensions in z-direction were about 19-30 μm depending on individual sample height and position (voxel-size: 418 × 418 × 800 nm). The Pinhole was kept constant at 72.65 μm (Airy 1-optimized value for this objective). No staining was needed since the spermathecal tubules are autofluorescent (370-770 nm).

For morphometrics, we randomly selected 15 images in each z-stack. Each z-stack represents one spermatheca and each image a section through it. One naive observer measured the outer perimeter and area of the spermathecal section, the total length of tubule walls within the section, and three tubule diameters at blindly selected positions. These measures were taken for a total of 225 images (15 images × 5 females × 3 strains). Volume was estimated as average area of spermathecal section × height of the stack (z-dimension). This approximates overall spermathecal volume, including both the volume that can be effectively used to store sperm (within tubules) and interstitial space between tubules. The latter seems minimal (Figure 2); tubules are tightly coiled and the entire spermatheca is contained in one sheath (Sinha, 1953). Complexity was estimated as the average total length of tubule walls per section divided by the average section area; this is comparable to the ratio of 'membrane length' per cell in stereobiology. Image analysis was carried out with NIH ObjectImage 1.61. The spermathecal walls appear to be chitinized. Indeed, spermathecal sizes are in the same range for mated and virgin females (GB and LA, unpubl. data).

Effect of interference with sperm storage on offspring paternity

Virgin females and males were obtained by isolating pupae from the stock populations and kept at a density of 6/vial (2.5 g medium, ø 1.0 cm, height 10 cm). At mating, beetles were aged 7 weeks post-eclosion. We doubly-mated 26 virgin wildtype females to a wildtype first male (focal male) followed by a Chicago black second male (competitor). We manipulated the interval between the first and second male to interfere with sperm storage processes in the female (Bloch Qazi et al., 1996). Focal males were marked with non-toxic paint to distinguish them from wildtype females. After the experiment, we measured male size (elytral length) to the nearest μm. Size (elytral length) of both males was assessed as a potential correlate of paternity success. The effect of size was not consistent in previous studies (e.g., significant in Lewis & Austad, 1990; nonsignificant in Lewis & Austad, 1994; Edvardsson & Arnqvist, 2000; Bernasconi & Keller, 2001).

We mated the males of our main experiment to virgin females of the same age as experimental females to measure egg production after single mating. After 18 h cohabitation in pairs in 2.5 g of medium, the male was removed and the female was allowed to lay eggs during 14 days. We recorded the number of adult progeny present in these vials 45 days after end of the oviposition period.

We interfered with the female's ability to control sperm storage by varying inter-mating interval. Females were moved to a petri dish containing her second male either immediately after the end of copulation with the first male (immediate remating, N = 13), or 24 h thereafter (24 h interval, N = 13). In the immediate remating group, all of 13 females remated, and remating occurred on average ± SD within 26 ± 13 (range 6-44) minutes from end of the previous copulation. Henceforth we refer to these treatment groups as to short and long inter-mating interval. Since remating rates are high in this species (Sokoloff, 1974), short inter-mating intervals may be common under natural conditions. All females were moved to new petri dishes (empty or containing the second male) at these two time points to ensure that females in all treatment groups experienced the same disturbance.

The mating arena consisted of a petri dish (ø 3.5 cm) lined with a filter paper. Females and males were first introduced singly in a mating arena and habituated for 30'. We observed copulations, recording the start of the experiment (time of placing male and female in the same arena), start and end of copulation, defined by intromission and occurrence of leg rubbing behaviour (Edvardsson & Arnqvist, 2000; Bloch Qazi, 2003). Mating observations were conducted at 25-28°C between 8:00 and 18:00 h (with replicates in randomized order) under a light source. Latency and copulation duration were used as covariables in the analysis. Previous studies showed that several intromission can occur within the same copulation (Bloch Qazi et al., 1996). Repeated intromission with the same female normally occurs, without significant effect on female fecundity or male paternity success (Lewis, 2004). To standardize this as far as possible, the focal male and the competing male were each allowed two intromissions. This resulted in overall copulation durations of on average 1.3-2.8 minutes for first males and

1.5-2.3 minutes for second males. Observations were done by simultaneously scanning up to 6 mating pairs. Replicates where one or both male did not achieve copulation, the female died, or measurements were missing (1 out of 26 replicates) were not included in the analysis of paternity.

Doubly-mated females were allowed to lay eggs during 3 days (72 ± 2 hours; 2.5 g medium). Females were transferred every three days to a new oviposition vials five times, and were kept for 6 days in the last (6th) ovipo-sition vial. Transferring the female to fresh oviposition medium ensures that the progeny develops in cohorts, which minimizes cannibalism (Lewis & Austad, 1990). This is important because juvenile mortality could otherwise bias P2-estimates assessed on adult progeny phenotype (Gilchrist & Partridge, 1997). On average, 121 ± 69 adult offspring were produced during these 21 days. We screened progeny phenotype to infer paternity 45 days after mid-oviposition. Females showing pure paternity of one of the two males were kept in the analysis. Exclusion of cases with pure paternity did not change significance of the results. Generation time under our laboratory conditions requires approximately 30 days (24-25 until the pupal stage, 6-7 days from nymph to adult).

Correlation of paternity with spermathecal traits

For a random subsample of 14 females out of the 25 replicates in which both males achieved copulation, we used confocal laser scanning microscopy to measure the outer perimeter and area of the spermatheca, the total length of tubule walls within sections, and three tubule diameters measured at blindly selected positions, as described above. Spermathecal volume was estimated as the average area of spermathecal section × height of the z-stack.

Statistical analyses were conducted with SPSS 10 for the Macintosh and R (Ihaka & Gentleman, 1996). Since a generalized linear model with binomial errors showed high overdispersion which could not be corrected by Williams' method (1982), paternity data in the experiment were analysed with standard ANOVA on total paternity obtained by the focal males (first male to mate) over the six oviposition periods, weighted by the ratio (number of progeny produced by focal female/mean number of progeny produced by all the females in the experiment). Since there was no evidence of heterogeneity of variances in the two treatment groups (F-test: 0.965, df = (11, 12), p = 0.946), the weighted data were analysed without further transformation.

Results

Intraspecific variation in spermathecal traits

Histologically, the spermatheca can be easily identified as a tubular structure containing sperm (Figure 1). Confocal microscopy revealed substantial variation among individuals in spermathecal shape as it results from coiling, length and width of tubules (Figure 2). Strains did not differ significantly in either average complexity, average tubule width or spermathecal volume (Table 1). Accordingly, variance components were 2-8 times larger within strains than among strains (Table 1D), indicating that variation among individuals is large. There was a trend (p = 0.086, Table 1C) for spermathecal volume to differ among strains. Interestingly, the spermathecal volume decreased from black, to reindeer and wildtype beetles, while body size increased in the same order (see Study species). Thus, the trend for differences in spermathecal volume among the three strains cannot be explained by an allometric relationship.

Effect of interference with sperm storage on offspring paternity

When remating was immediate, the observed paternity of the focal male (weighted mean of observed values ± SE: 27% ± 10%) was lower than when the female was exposed to the second male 24 h later (40% ± 11%), but this difference was not found to be significant, as indicated by Table 2.

There was no significant difference in the total number of progeny produced by females over 21 days between treatment groups (short interval: 101 ± 18, long interval: 123 ± 19, t = 0.843, df = 24, p = 0.407).

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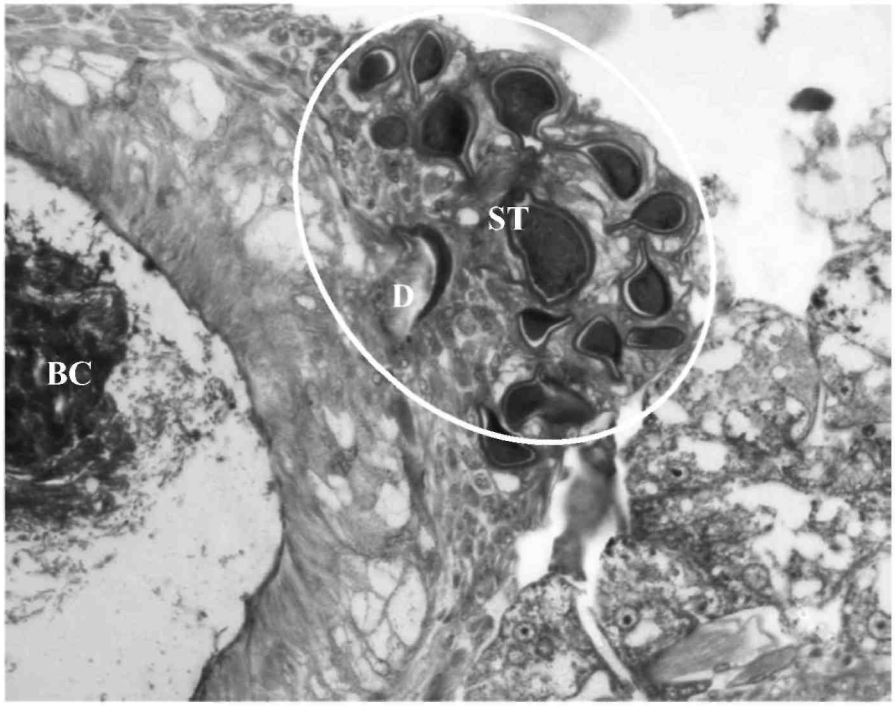
Figure 1. *Histological section through the spermatheca (ST) of Tribolium castaneum showing tubules, the bursa copulatrix (BC) and the main duct (D) between BC and ST. Sperm cells are visible in the BC and the ST tubules.*



Correlation of paternity with spermathecal traits

We analysed the correlation of residuals from the above analysis (in Table 2) with potential covariables. None of these variables correlates significantly with paternity (Table 3). Quadratic effects of copulation duration (as reported by Edvardsson & Arnqvist, 2000) were also not significant. None of the spermathecal traits was significantly correlated with the residuals of paternity success of the focal male from Table 2.

Discussion

Intraspecific variation in spermathecal traits

We found substantial variation in spermathecal volume, complexity and tubule width in female flour beetles from three laboratory strains. Large variation in spermathecal volume has also been independently reported for Chicago black females (Fedina & Lewis, 2004). Most of spermathecal trait variation was at the level of the individual rather than among the three strains. Similarly, examining ejaculate size and sperm lenght in 14 populations of *T. castaneum* (Arnaud et al., 2001), found that these traits were more variable within rather than between populations. We currently do not know what maintains variation in spermathecal morphology, and to what extent this variation is heritable.

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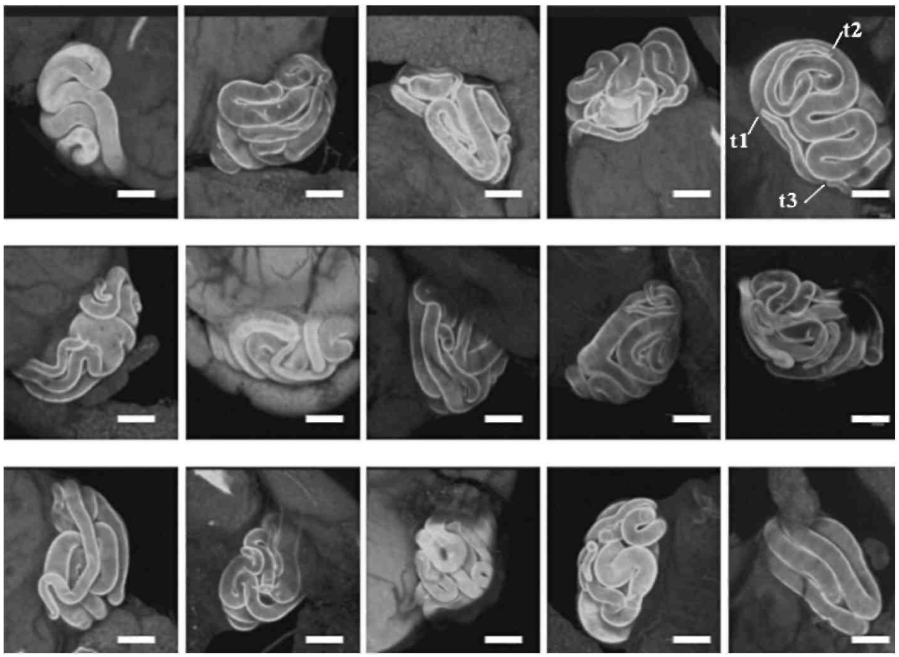
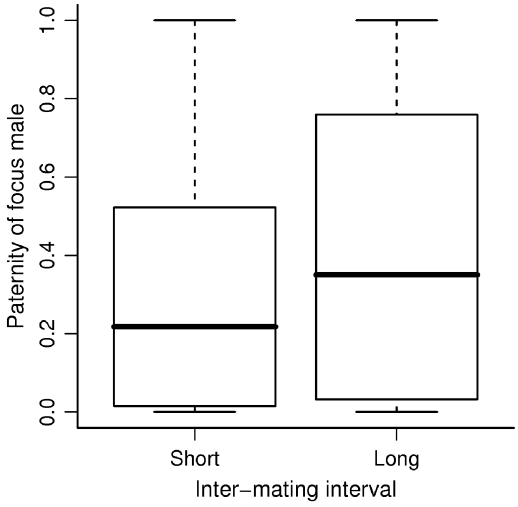
Figure 2. *Confocal laser scanning micrographs (maximum projection) showing variation in Tribolium castaneum spermatheca (N = 12 females; white bars = 40 µm).*



It is possible that variation among females in spermathecal morphology explains at least partly the large variation in sperm competition success in this species (see below; Lewis & Austad, 1990; Fedina & Lewis, 2004). The idea that the size and shape of sperm storage organs (Walker, 1980) as well as their complexity (Hellriegel & Ward, 1998) may be important in determining paternity is still largely unexplored. In the yellow dung fly, the three spermathecae of a female are not homogeneously filled during copulation (Hosken et al., 1999), and this leads to different proportional representation of the second male's sperm across spermathecae of the same female (Hellriegel & Bernasconi, 1999). The study on dung flies showed that spermathecal complexity (multiple compartments) are used to store the sperm of different males in different proportions, yet it did not investigate the role of spermathecal volume and shape. Because of recent reports of male-female coevolution of reproductive traits across species (Hi-hara & Kurokawa, 1987; Presgraves et al., 1999; Morrow & Gage, 2000; Miller & Pitnick, 2002; Koene & Schulenburg, 2005), more attention may be devoted also to the function of intraspecific variation in spermathecal morphology in the future.

Figure 3. *Box and Whisker's plot of paternity of focal males (first male to mate) when females remated immediately (i.e., within 2 hours of first mating) or after 24 hours*.



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Effect of interference with sperm storage on offspring paternity

We tested whether sperm storage is the mechanism of cryptic female choice, and based on a priori-knowledge on the dynamics of sperm storage (Bloch Qazi et al., 1998) we expected paternity to depend on inter-mating interval and to be correlated with spermathecal morphology. Specifically, we expected this correlation and female control of paternity to be stronger for longer than for shorter inter-mating intervals.

Although the focal male (first to mate) achieved shorter paternity when re-mating was immediate, as expected from last-male advantage in this species (Lewis & Austad, 1990) and consistent with studies of the dynamics of sperm storage and sperm elimination after mating (Bloch Qazi et al., 1996), this difference was not significant. It would be interesting in future studies to investigate more closely the effect of inter-mating interval on paternity in this species, although this will require a large sample size due to large variation in paternity values.

We did not find any significant evidence for a correlation between spermathecal morphology and paternity. In a similar, independent study (Fedina & Lewis, 2004) total spermathecal volume and paternity achieved by the second male to mate (P2 value) were negatively correlated in a sample of 18 females with a short remating interval (≤ 50 minutes). The difference between the results of the two studies, namely lack of significance in our study, may be explained by differences between the female strains used (Chicago black in Fedina and Lewis, 2004, Ga-1 wildtype in our study), by differences in experimental design (CO_2-anaesthetization in Fedina & Lewis, 2004; variation in intermating intervals in the present study), or by statistical power (18 females in Fedina & Lewis, 2004, vs 14 females in our subsample for confocal microscopy).

Table 1. *Univariate analyses of variance for spermathecal traits in three Tri-bolium castaneum strains (wildtype Ga-1, Reindeer, Chicago black). Covariate: exact zoom factor.*

A. Dependent variable: average complexity					
Source	Sum of squares	df	Mean square	*F*	*P*
Covariate	0.783	1	0.783	0.714	0.416
Strain	0.894	2	0.447	0.407	0.675
Error	12.070	11	1.097		
B. Dependent variable: average tubule width					
Source	Sum of squares	df	Mean square	*F*	*P*
Covariate	87.28	1	87.28	2.14	0.171
Strain	148.433	2	74.216	1.820	0.208
Error	448.676	11			
C. Dependent variable: volume (type A, see Methods)					
Source	Sum of squares	df	Mean square	*F*	*P*
Covariate	8.89	1	8.89	5.517	0.039
Strain	9.982	2	4.991	3.096	0.086
Error	17.73	11			
D. Variance components*					
			Complexity	Tubule width	Volume
Variance among strains			-0.14	7.24	7.32
Variance among females within strains			1.10	40.79	16.12

** ANOVA Type I Sum of squares: negative values are possible for variance components that do not differ significantly from zero.*

Table 2. *Analysis of variance for the effect of inter-mating interval on paternity success of focal males (cumulative P1-value over six oviposition intervals), weighted by number of progeny produced by each female over the average of all females.*

Source	**Sum of squares**	**df**	**Mean square**	***F***	***P***
Interval	0.1171	1	0.1171	0.8496	0.366
Residuals	3.1712	23	0.1379		

Table 3. *Pairwise Pearson's correlation coefficients between the residuals from Table 2 and potential covariables.*

	r	***N***	***P***
Duration first mating	-0.068	25	0.748
Duration second mating	0.252	22	0.257
Latency second mating	0.025	21	0.912
Latency first mating	0.179	24	0.403
Elytral length of male 1	0.040	23	0.857
Elytral length of male 2	-0.140	24	0.512
Spermathecal volume	-0.219	14	0.452
Spermathecal complexity	-0.410	14	0.146

In conclusion, we found substantial intraspecific variation in spermathecal morphology, but no significant correlation of this variation with paternity in doubly-mated females. Also, we found that short inter-mating intervals favour the first male compared to long inter-mating intervals, but this difference was not significant.

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